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Development of tolerance in D3 dopamine receptor signaling is accompanied by distinct changes in receptor conformation

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ABSTRACT

The D3 but not D2 dopamine receptors exhibit a tolerance property in which agonist-induced D3 receptor response progressively decreases upon repeated agonist stimulation. We have previously shown that the D3 receptor tolerance property is not mediated by receptor internalization, persistent agonist binding or a decrease in receptor binding affinity. In this paper, we test the hypothesis that alterations in D3 receptor conformation underlie the tolerance property. Structural models of wild type and mutant human D3 receptors were generated using the beta adrenergic receptor crystal structure as a template. These models suggested that the agonist-bound D3 receptor undergoes conformational changes that could underlie its tolerance property. To experimentally assess changes in receptor conformation, we measured the accessibility of native cysteine residues present in the extracellular and transmembrane regions of the human D3 receptor to two different thiol-modifying biotinylating reagents. The accessibilities of the native cysteine residues present in the D3 receptor were assessed under control conditions, in the presence of agonist and under conditions that induced receptor tolerance. By comparing the accessibility of D3 receptor cysteine residues to hydrophobic and hydrophilic thiol-modifying biotinylating reagents, we show that the alteration of D3 receptor conformation during tolerance involves the net movement of cysteine residues into a hydrophobic environment. Our results show that the conformation state of the D3 receptor during tolerance is distinct from the conformation under basal and agonist-bound conditions. The results suggest that the D3 receptor tolerance property is mediated by conformational changes that may uncouple the receptor from G-protein signaling.

1. Introduction

The dopamine receptors include D1 and D5 receptor subtypes (D1-like) and D2, D3, and D4 receptor subtypes (D2-like). D2 and D3 receptors modulate various signal transduction pathways via associated G-proteins [1]. Agonist stimulation of D2 and D3 receptors inhibits adenylyl cyclase and decreases cAMP production via Gαi/o subunit [2]. D2 and D3 receptors also couple to the mitogen-activated protein kinase (MAPK) pathway [3–5] and G-protein coupled inward rectifier potassium (GIRK) channels [6,7] via the βγ subunit of the heterotrimeric G-proteins.

By comparing the coupling of D2 and D3 dopamine receptors to endogenous GIRK channels and other effectors in AtT-20 cells, we previously demonstrated that the human D3, but not the D2 dopamine receptor exhibits tolerance and slow response termination properties [8,5] (Fig. 1). The D3 receptor tolerance property is defined as a progressive decrease in agonist-induced GIRK response upon repeated agonist stimulation. Typically, the magnitude of the second agonist-induced GIRK response is reduced by 60% compared to the first GIRK response [8,5] (Fig. 1). The slow response termination property of the D3 receptor describes the prolonged delay in termination of agonist-induced GIRK response after the removal of the agonist. The response termination rate of D3 receptor-induced GIRK response was 15-fold slower than the D2 receptor-induced GIRK response [8]. Together the tolerance and slow response termination properties distinguish D3 and D2 receptor signaling, which could be potentially important for normal physiological function. We have previously shown that the D3 receptor tolerance property is not mediated by receptor internalization, persistent agonist binding or a decrease in receptor binding affinity [8,5]. In this paper, using receptor modeling studies, we

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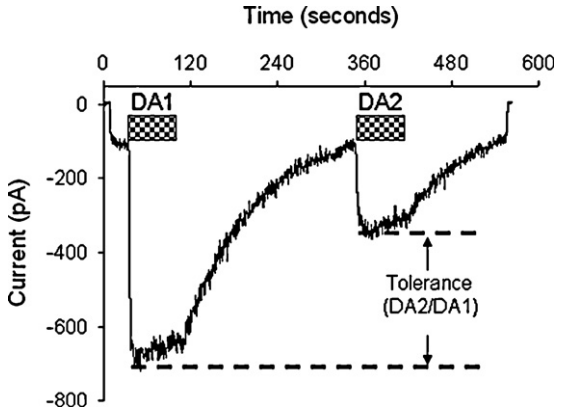

Fig. 1. Dopamine-induced tolerance. Representative current traces from whole cell voltage clamp recording of AtT-D3 cell. The cell was held at −65 mV and inward currents were elicited by two 1 min applications of 100 nM dopamine (DA, hatched rectangles). Tolerance is quantified as the ratio of second to first DA-induced GIRK current response.

determined that the conformational state of the wild type D3 receptor, which exhibits tolerance, is significantly different from a mutant D3 receptor that does not show tolerance. Similarly the conformational state of the wild type D3 receptor to tolerance- and non-tolerance-causing ligands are significantly different. These results led us to test the hypothesis that alterations in D3 receptor conformation underlie its tolerance property. To assess changes in receptor conformation we used a novel approach and measured the accessibilities of native cysteine residues present in the extracellular and transmembrane (TM) regions of the human D3 receptor to two different thiol-modifying biotinylating reagents (MTSEA- and TS-biotin-XX). The accessibilities of the native cysteine residues present in the D3 receptor were assessed under control conditions, in the presence of agonist and under conditions that induced receptor tolerance. The results show that the conformation state adopted by the D3 receptor, reflected by the relative alterations in cysteine accessibilities, is different under the three conditions. The results were confirmed by testing the cysteine accessibilities in a mutant D3 receptor that lacks tolerance and by using a D3 receptor agonist that does not elicit tolerance and slow response termination properties in wild type receptors. By comparing the accessibilities of D3 receptor cysteine residues to hydrophobic and hydrophilic thiol-modifying biotinylating reagents, we showed that the alteration of D3 receptor conformation during tolerance involves the net movement of cysteine residues into a hydrophobic environment. Together our results demonstrate that, unlike other dopamine receptor subtypes and many GPCRs, the termination of D3 receptor signaling is achieved by an alteration of receptor conformation.

2. Materials and methods

2.1. Cell culture and transfection

AtT-20 mouse pituitary cells were grown in Ham's F10 medium with 5% fetal bovine serum (FBS), 10% heat-inactivated horse serum, 2 mM glutamine and 50 μg/ml gentamicin (Invitrogen, Carlsbad, CA). AtT-20 cells stably expressing Flag™-tagged wild type D3 (AtT-Flag™ D3) or mutant C147K D3 (AtT-Flag™ D3C147K) dopamine receptor subtypes were maintained in the above F10 culture media supplemented with 500 μg/ml G418 (Invitrogen). For electrophysiological characterization, cells were plated onto glass coverslips coated with 40 μg/ml poly L-lysine (Sigma, St. Louis, MO) in a 12-well plate. All electrophysiological characterizations were carried out 36–48 h post-plating.

2.2. Electrophysiology, drugs, and solutions

Agonist-activated currents were measured by the whole cell patch clamp technique as described before [8]. Briefly, cells were held at −65 mV and inward K^+ currents induced by drug solutions were measured. The standard external solution (SES) used for K^+ current measurements was, in mM: 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes [pH 7.4], and 10 glucose and the pipette solution contained in mM: 130 K-aspartate, 20 NaCl, 1 $MgCl_2$, 10 Hepes [pH 7.4], 10 glucose, 0.1 GTP, 5 Mg-ATP, and 1 EGTA. To enhance inwardly rectifying K^+ currents, controls and drug exposures were performed in solutions with elevated extracellular [K^+] (30 mM) by substitution for Na^+. Quinpirole and cis-8-hydroxy-3-(n-propyl)-1,2,3a,4,5,9b-hexahydro-1H-benz[e]indole hydrobromide (PBZI; Sigma) were dissolved in water and used at indicated concentrations. A 10 mM stock of dopamine (Sigma) was freshly dissolved in 100 mM ascorbic acid and used at a final concentration of 100 nM. Drug solutions were delivered to cells via a multi-barreled micropipette array. The current responses were normalized to the cell capacitance, to account for variation in cell size.

2.3. Data analyses

Currents were measured using an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA) and sampled through a Digidata 1322A interface (Axon Instruments) using the pClamp 8.0 software (Axon Instruments). Data files were imported into SigmaPlot (SPSS Inc., Chicago, IL) for analysis and display. SigmaPlot was also used to perform two-tailed Student's *t*-test. Analysis of variance (ANOVA) and the Holm's multiple pair-wise comparison tests were performed with Primer of Biostatistics software (Version 5.0, McGraw-Hill, New York, NY). Data were considered statistically different when probability was <0.05.

2.4. Biotin labeling of cysteine residues and immunoblotting of cell membrane proteins

AtT-Flag™ D3 or AtT-Flag™ D3C147K cells were harvested and treated with: (A) standard external solution (SES) for 1 min, or (B) pretreated with quinpirole or PBZI for 1 min, or (C) pretreated with quinpirole or PBZI for 1 min and then washed with SES for 5 min to remove agonists. Following the three treatment conditions, the cells were treated with four different concentrations of N-biotinylamino-ethylmethanethiosulfonate-XX (MTSEA-biotin) or biotin-XX ethylenediamine thiosulfate (TS-biotin) (Biotium, Hayward, CA) for 2 min. The XX-linker is incorporated to facilitate biotin–streptavidin interaction following the thiol modification. Stock solutions of MTSEA-biotin (50 mM) and TS-biotin (100 mM) were freshly prepared just before use in anhydrous DMSO (Sigma, catalog number 276855). Working stock solutions were prepared by further diluting the stock solutions in anhydrous DMSO. The concentrations of the working stock solutions were such that each treatment condition had the same final concentration of DMSO. The treatment scheme details are shown in Fig. 8. The concentrations of MTSEA/TS-biotin used were 0.02, 0.04, 0.06, and 0.1 mM. Excess MTSEA/TS-biotin was removed by washing the cells two times with SES. The final concentration of thiol biotinylating reagents were selected following extensive optimization experiments in which we varied the concentration of the biotinylating reagents from 0.01 mM to 1 mM and treated the cells for 1–10 min. Longer incubations and higher concentrations resulted in saturation under control conditions. The MTSEA/TS-biotin concentrations and time of treatment that were selected resulted in a consistent linear increase in biotinylated D3 proteins under control conditions.